Rapidly assessing changes in bone mineral balance using natural stable calcium isotopes

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The ability to rapidly detect changes in bone mineral balance (BMB) would be of great value in the early diagnosis and evaluation of therapies for metabolic bone diseases such as osteoporosis and some cancers. However, measurements of BMB are hampered by difficulties with using biochemical markers to quantify the relative rates of bone resorption and formation and the need to wait months to years for altered BMB to produce changes in bone mineral density large enough to resolve by X-ray densitometry. We show here that, in humans, the natural abundances of Ca isotopes in urine change rapidly in response to changes in BMB. In a bed rest experiment, use of high-precision isotope ratio MS allowed the onset of bone loss to be detected in Ca isotope data after about 1 wk, long before bone mineral density has changed enough to be detectable with densitometry. The physiological basis of the relationship between Ca isotopes and BMB is sufficiently understood to allow quantitative translation of changes in Ca isotope abundances to changes in bone mineral density using a simple model. The rate of change of bone mineral density inferred from Ca isotopes is consistent with the rate observed by densitometry in long-term bed rest studies. Ca isotopic analysis provides a powerful way to monitor bone loss, potentially making it possible to diagnose metabolic bone disease and track the impact of treatments more effectively than is currently possible.

osteopenia | biomarker | medical geology | biosignature | spaceflight

In humans and most other vertebrates, bone is continuously replaced through remodeling, resulting from the coupled actions of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). In healthy adults, the rates of bone formation and resorption are equal, and therefore, net bone mineral balance (BMB) is near zero. Disruptions in BMB caused by diseases such as osteoporosis, multiple myeloma, and some metastatic cancers can have serious or even fatal consequences. Sensitive methods to directly measure BMB could benefit research and clinical practice in the study and treatment of bone disease.

The abundances of the naturally occurring isotopes of Ca in urine and soft tissue (blood and nonbone tissues) should be sensitive to changes in BMB. A large body of research establishes that these six isotopes (^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca, and ^{48}Ca) react at different rates depending on atomic mass and that these differences fractionate the isotopes, producing variations in Ca isotope abundances in nature (1–3). In the body, changes in BMB should lead to variations in the abundances of Ca isotopes for two related reasons, one tied directly to bone metabolism and the other indirectly by the excretion of Ca through the kidneys. Both processes should shift Ca isotope abundances in urine in the same direction in response to changes in BMB.

First, in vertebrates, bone formation favors the lighter isotopes of Ca (4, 5), thus depleting soft tissue of light Ca isotopes. Bone resorption releases this isotopically light Ca back into soft tissue with little or no isotope selectivity. As a result of this difference in isotope fractionation, the Ca isotope abundances in soft tissue should shift to heavier Ca isotope values when BMB is positive (bone formation exceeds resorption) and lighter values when BMB is negative (bone resorption exceeds formation) (4). Urinary Ca is derived from soft tissue. Therefore, shifts in soft tissue Ca isotopes should be reflected as correlated shifts in the Ca isotope composition of urine.

Second, Ca isotopes are also fractionated during excretion of Ca in the kidneys. Comparison of Ca in urine and soft tissue indicates that heavy isotopes are preferentially excreted (6). Although the mechanism of this renal Ca isotope effect is not well understood, Ca isotope abundances in soft tissue should shift to heavier Ca isotope values when BMB is positive and Ca excretion rate decreases and lighter values when BMB is negative and Ca excretion rate increases (see *Discussion*). As with the bone metabolism effect, such shifts in the Ca isotope composition of soft tissue should be reflected in the Ca isotope composition of urine. Consistent with this expectation, a significant inverse correlation has been observed between the Ca concentration of urine and its isotopic composition (7).

Two independent bed rest studies have confirmed that Ca isotope abundances in urine shift to lighter values when BMB is negative (7, 8), which was expected from the two processes described above. Bed rest induces bone loss because of skeletal unloading, and it is used to model the effects of space flight on human bone metabolism. In samples from four subjects enrolled in a 17-wk bed rest study, variations in Ca isotope abundances in urine were consistent with results from established collagen cross-link assays and X-ray densitometry indicating net bone resorption (8). In preliminary data from a 35-d bed rest study (7), similar variations in Ca isotope abundances in urine were observed in samples from seven subjects.

We report here a more detailed evaluation of Ca isotope variations from a 30-d bed rest study. This study was designed to assess how rapidly the Ca isotope signal appears and evaluate its potential as a quantitative measure of bone loss. The study included 12 participants monitored for 12 d before bed rest, 30 d during bed rest, and 7 d after bed rest. A 10-d diet rotation was controlled and consistent for all participants during the duration of the study. Urine samples were collected at time points throughout the study to examine short-term variations in Ca isotope abundances either from analyses of 24-h pooled samples or in some cases, on all individual voids throughout the day.

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Conflict of interest statement: Donald DePaolo served on the PhD dissertation committee for J.L.S. from 1997 to 2000, and J.L.S. and Donald DePaolo coauthored two papers on calcium isotopes (1997 and 1999). Some of the techniques used in this research are covered under patent claims being pursued by Arizona Technology Enterprises (AzTE), the technology transfer office of Arizona State University.

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N-telopeptide (NTx; a marker of bone resorption) was measured in select urine samples. Bone-specific alkaline phosphatase (BSAP; a biochemical marker of bone formation) was measured in serum samples from eight blood collections throughout the study.

We present high-precision characterization of Ca isotope abundance in ~200 urine samples. Urine samples were given priority over blood; higher Ca concentrations in urine compared with blood ease analytical challenges, and urine samples are easier to obtain from subjects. This number of sample analyses is not practical with the time-consuming mass spectrometric methods used in previous studies (6–8). Therefore, we applied a validated method that uses multicollector inductively coupled plasma source mass spectrometry (9).

Results

Data are presented as $\delta^{44/42}$Ca relative to a standard ICP1 (9) in parts per ten thousand (pptt) units [$\delta^{44/42}Ca = ([^{44}Ca/^{42}Ca]_{sample}/[^{44}Ca/^{42}Ca]_{standard} - 1) \times 10{,}000$]. This notation follows the new International Union of Pure and Applied Chemistry convention for high-precision measurements of natural isotope variations (10).

Urinary $\delta^{44/42}$Ca of subjects entering the study ranged from −0.3 to 7.8 pptt (1 SD = 2.5 pptt) (Table S1). To permit comparison between individuals, and because the primary parameter of interest is the change from the onset of bed rest, each subject's $\delta^{44/42}$Ca was referenced to their individual average pre-bed rest baseline $\delta^{44/42}$Ca value (determined as the average of $\delta^{44/42}$Ca from days −12 to −1). During the pre-bed rest period, the $\delta^{44/42}$Ca of each subject varied by up to 0.9 pptt (1 SD). There was no significant relationship between pre-bed rest urinary $\delta^{44/42}$Ca and time, body mass index, sex, or age.

Between days 7 and 30 of bed rest, the $\delta^{44/42}$Ca value for all subjects decreased by ~2 pptt and remained low during the remainder of bed rest and into the post-bed rest period ($P < 0.001$) (Fig. 1*A* and Table 1). A generalized estimating equation was used to examine the significance of the Ca isotope variations while properly accounting for the tendency for multiple samples from each subject to cluster. By day 10 of bed rest, the average baseline-normalized $\delta^{44/42}$Ca during bed rest was significantly lower than the pre-bed rest mean ($P = 0.043$).

In addition, we observed a significant correlation between urinary $\delta^{44/42}$Ca and Ca concentration ($r^2 = 0.45$, $P < 0.0001$) (Fig. S1*A*) and between urinary $\delta^{44/42}$Ca and total urinary Ca excretion rate ($r^2 = 0.48$, $P < 0.0001$) (Fig. S1*B*). Total urinary Ca excretion was calculated from the weight and Ca concentration of 24-h urine pools.

Discussion

$\delta^{44/42}$Ca and BMB. The systematic decrease in urinary $\delta^{44/42}$Ca after the start of bed rest (Fig. 1*A* and Table 1) is consistent with the expectation that the onset of negative BMB shifts the isotopic composition of Ca in urine to lighter values. Bone biochemical markers support this interpretation. Resorption markers can respond quickly to changes in bone biology, because differentiation of osteoclasts occurs after just 4 d in vitro (11). Biomarkers of bone resorption, including NTx and C-telopeptide, have been observed in urine and serum after just 2 d of bed rest (12). Here, NTx increased by day 9 of bed rest ($P < 0.001$), the first analysis after initiation of bed rest (Fig. 1*B* and Table 1). Throughout bed rest and into the post-bed rest period, the NTx signal remained significantly elevated relative to pre-bed rest, indicating that bone resorption increased.

BSAP (a biochemical marker of bone formation) did not change significantly during bed rest ($P = 0.52$) (Fig. 1*C* and Table 1). This result is consistent with current understanding of bone physiology and results of previous bed rest and space flight experiments. Osteoblast cells can take up to 30 d to differentiate, and therefore, a significant increase in bone formation rates was not expected on the timescale of this study (13). Moreover, in the absence of heavy resistive exercise, bone formation rates generally do not change during bed rest or space flight (14).

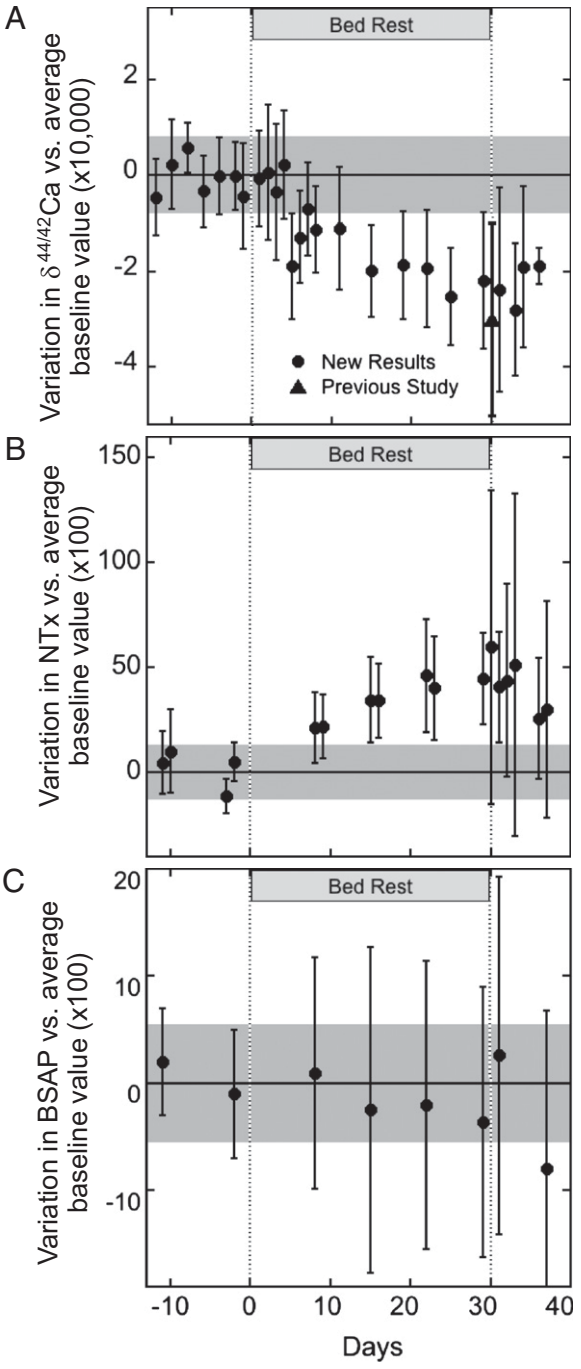

Fig. 1. Variation in Ca isotopes and biomarkers before, during, and after bed rest. Changes were calculated as the difference between the measured value at each time point and the average of the pre-bed rest values (baseline) for that individual. Gray bar that includes zero is 1 SD of pre-bed rest values. (*A*) Change in urinary Ca isotopes during bed rest in parts per ten thousand. All values are mean ± 1 SD of all difference determinations from that time point ($n = 12$). The 25 data points represent Ca isotope abundance determination in more than 200 individual samples. The value from the previous published bed rest study (7) is plotted for comparison. (*B*) Percentage change from baseline in concentration of NTx, a bone resorption biomarker (mean ± 1 SD, $n = 12$). (*C*) Percentage change from baseline in concentration of BSAP, a bone formation biomarker (mean ± 1 SD, $n = 12$).

Table 1. Summary of data for 12 subjects in bed rest

Biomarker	Unit	Pre-bed rest (days −14 to 0) mean ± 1 SD; *N*	Bed rest (days 7–30) mean ± 1 SD; *N*	*P*
BSAP	U/L	25.7 ± 8.2; 24	24.85 ± 6.5; 48	0.52
NTx	nmol/d	539 ± 218; 49	742 ± 324; 91	<0.001
$\delta^{44/42}$Ca	pptt	2.4 ± 2.5; 47	0.9 ± 2.3; 116	<0.001

P values represent the significance of the difference between the populations of data obtained during days −14 to 0 and days 7–30. BSAP, bone-specific alkaline phosphatase; *N*, number of samples pre-bed rest and during bed rest (post-bed rest samples are not included here); NTx, N-telopeptide; pptt, parts per ten thousand.

As inferred from Ca isotope and concentration data in the study by Heuser and Eisenhauer (6), we found that urinary $\delta^{44/42}$Ca is inversely correlated with Ca concentration and total urinary Ca excretion rate (Fig. S1). This correlation suggests that at least some of the lightward shift in urinary $\delta^{44/42}$Ca observed after the start of bed rest resulted from an increase in Ca excretion arising from enhanced bone resorption. The relative importance of this renal isotope effect, which is indirectly linked to change in BMB, vs. the direct isotope effect from bone biology, is assessed in the next section.

Unlike the study by Heuser and Eisenhauer (6), we did not see a correlation between age and urinary $\delta^{44/42}$Ca. This difference in results is not surprising given the differences in study designs. The work by Heuser and Eisenhauer (6) compared the urinary Ca isotope composition of a young boy in active skeletal growth with an elderly woman of an age at which rapid bone loss is common. The ages of all participants in the present study were such that their bone mineral mass should be relatively stable. Therefore, no such relationship was expected.

Subjects entering this study showed a range of 8.1 pptt in urinary $\delta^{44/42}$Ca. Few data on normal variation in urinary $\delta^{44/42}$Ca are available for the human population. $\delta^{44/42}$Ca variation of 8.1 pptt is within the range of $\delta^{44/42}$Ca measured for common dietary sources of Ca (4, 15) and therefore part of the background variation could result from differences in dietary history. Metabolic and physiological variation, such as in Ca absorption, renal Ca excretion rate, or efficiency, may also contribute to intraindividual variability (16–20). Additional research is needed to determine the causes of the variation observed in the pre-bed rest background urinary $\delta^{44/42}$Ca.

However, the observed shift in $\delta^{44/42}$Ca during bed rest cannot be ascribed to changes in dietary Ca or other uncontrolled, systemic environmental signals during the study. Although the Ca isotope composition of the study diet has not yet been measured, participants were fed the same diet, and therefore, the average Ca isotope intake over the 10-d menu rotation was the same. Subjects started the bed rest study on different calendar dates, and the 10-d diet rotation did not correspond to the sampling schedule.

$\delta^{44/42}$Ca differs importantly from biochemical markers such as NTx and BSAP in that the change in $\delta^{44/42}$Ca from baseline provides a direct indication of change in BMB. This follows because the abundances of Ca isotopes in urine and soft tissue represent the net effect of changes in bone formation and resorption and Ca excretion integrated over the 2- to 3-d residence time of Ca in soft tissue compartments (21, 22). In contrast, each biochemical marker reflects only one process (either bone resorption or formation). Mathematically combining these biochemical markers into a single quantitative measure of net BMB has not been and may not be possible, because neither marker can be quantitatively related to the rate of the process that it measures, let alone to the other marker (23, 24). The application and interpretation of biochemical markers are also confounded by high interlaboratory analytical variability (25). However, interlaboratory variation of $\delta^{44/42}$Ca is within the analytical uncertainty for any single laboratory (9, 26), which permits direct comparison between isotopic measurements made by different laboratories.

At present, X-ray densitometry is the definitive way to monitor changes in BMB (27). Monitoring changes in urine $\delta^{44/42}$Ca seems to be substantially more sensitive than X-ray densitometry. Using the Ca isotope method, variations in BMB were detectable within 10 d of the start of bed rest compared with the 12 wk required for bed rest to produce a change in bone mineral density detectable by bone X-ray densitometry (28).

Quantifying Bone Loss. Because $\delta^{44/42}$Ca is directly related to net BMB, it should be possible to use Ca isotopes to quantitatively assess the extent of bone loss (or gain). To illustrate this potential, we used an updated isotope mass balance model based on models developed in the works by Skulan and DePaolo (4) and Heuser and Eisenhauer (6). The model quantifying the amount of bone lost during the 30-d bed rest study is depicted in Fig. 2. This model uses the difference in Ca isotopic composition of urine before and during bed rest to estimate the rate of bone loss. It is similar to the model by Heuser and Eisenhauer (6) in that it explicitly includes renal Ca isotope fractionation. We have also extended the model to explicitly include hepatic Ca isotope fractionation. However, we show that hepatic isotope fractionation is unlikely to affect estimates of bone loss, because this flux is not expected to vary with changes in BMB (*Materials and Methods*).

In addition to measurements of $\delta^{44/42}$Ca, the model requires that we estimate values for the fractionation factor of Ca isotopes during bone mineralization ($\varepsilon^{44/42}Ca_{bone}$), the renal Ca

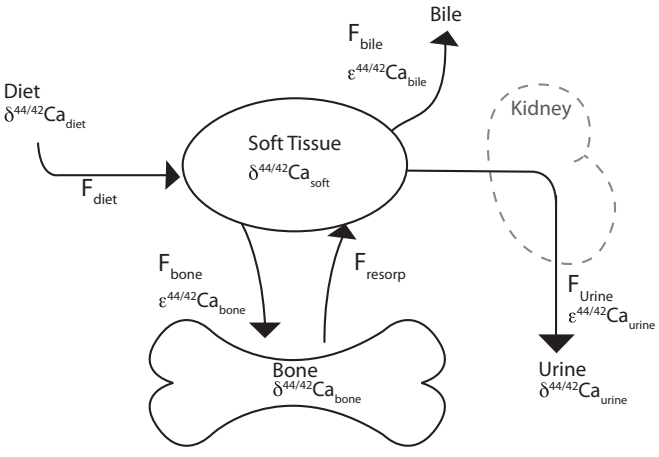

Fig. 2. Schematic of model pools and fluxes, including the isotopic fractionations for various transformations. F_{diet} is the flux of calcium absorbed from diet, F_{bone} and F_{resorp} are the bone formation and resorption fluxes, and F_{urine} and F_{bile} are the net excretion fluxes of calcium through urine and bile, respectively. $\delta^{44/42}Ca_{soft}$ and $\delta^{44/42}Ca_{bone}$ are the Ca isotopic compositions of the soft tissue and bone pools, $\delta^{44/42}Ca_{diet}$ and $\delta^{44/42}Ca_{urine}$ are the isotopic compositions of the fluxes of Ca absorbed from diet and excreted in urine, and $\varepsilon^{44/42}Ca_{bone}$, $\varepsilon^{44/42}Ca_{urine}$, and $\varepsilon^{44/42}Ca_{bile}$ are the isotopic fractionations associated with forming bone, urine, and bile, respectively, from the soft tissue pool.

isotope fractionation factor ($\varepsilon^{44/42}Ca_{urine}$), the mass of skeletal Ca (M_{bone}), the background rate of skeletal remodeling (F_{bone}), and the rate of intestinal absorption of dietary Ca (F_{diet}).

- $\boldsymbol{\varepsilon^{44/42}Ca_{bone}}$. We estimated the fractionation factor of bone formation to be 7.5 ± 1 pptt (1 SD) and based the estimate on the offset between bone and diet in terrestrial vertebrates (4, 5). This value seems to be similar among a phylogenetically wide range of vertebrates (4, 5) and is unlikely to vary between individuals.
- $\boldsymbol{\varepsilon^{44/42}Ca_{urine}}$. Renal Ca isotope fractionation is an important but poorly constrained term (6). Soft tissue tend to have a Ca isotopic composition similar to the composition of diet, whereas urine tends to be isotopically heavy (4, 6, 8). For example, $\delta^{44/42}$Ca of all urine samples from a 17-wk bed rest study were higher than the dietary mean by an average of 9 pptt (8). In data from an animal model, $\delta^{44/42}$Ca of urine collected from a sow was 11.4 pptt higher than $\delta^{44/42}$Ca of simultaneously collected serum (Table S2). These values are nearly identical to the values estimated or measured in the work by Heuser and Eisenhauer (6) (9.6 pptt for humans and 10 pptt for sows). Together, these data indicate that the renal fractionation is almost certainly positive and that $\varepsilon^{44/42}Ca_{urine} \sim 10$ pptt.
- $\boldsymbol{M_{bone}}$. Assuming an average skeletal mineral mass of 3,000 g (29) and a Ca content in bone mineral of 32.2% (30), we estimate that ~1,000 ± 100 g Ca is stored in the average human skeleton.
- $\boldsymbol{F_{bone}}$. We estimated the background rate of skeletal remodeling to be 500 ± 100 mg Ca/d based on two studies. In the first study, Smith et al. (21) estimated rates of new bone formation of 190–635 mg Ca/d for male and female volunteers. In a follow-up study, the work by Smith et al. (22) estimated the rate of bone formation of 16 astronauts at 490 ± 153 mg Ca/d before spaceflight and 434 ± 194 mg Ca/d during spaceflight.
- $\boldsymbol{F_{diet}}$. The average measured dietary Ca intake during bed rest for all subjects in this study was 1,450 ± 170 mg Ca/d. Accounting for the bioavailability of foods in the typical diet (31), we estimated that 390 ± 96 mg Ca/d was absorbed in the intestines.

The model-derived relationships between the $\delta^{44/42}$Ca shift of urine during bed rest and BMB based on these parameters are shown in Fig. 3. The relationships permit a straightforward extrapolation of the rate of bone loss from a measured shift in $\delta^{44/42}$Ca. Because the exact value of $\varepsilon^{44/42}Ca_{urine}$ (the renal fractionation) has not yet been directly measured in humans, curves for $\varepsilon^{44/42}Ca_{urine} = 0$ and $\varepsilon^{44/42}Ca_{urine} = 10$ pptt are shown to give a sense of the parameter uncertainty. It is apparent that, as renal fractionation increases, a given shift in urinary $\delta^{44/42}$Ca corresponds to a smaller change in BMB. Hence, the net effect of renal fractionation is to amplify variations in urinary $\delta^{44/42}$Ca in response to changing BMB. This finding increases the sensitivity of the $\delta^{44/42}$Ca technique for detecting variations in BMB. This increase in sensitivity is greatest as renal fractionation rises from 0 to about 5 pptt. Greater increases add relatively little additional sensitivity, and therefore, uncertainty in the value of $\varepsilon^{44/42}Ca_{urine}$ adds minimal uncertainty to the calculation of BMB as long as renal fractionation in humans is above about 5 pptt (which is likely) (Fig. 4).

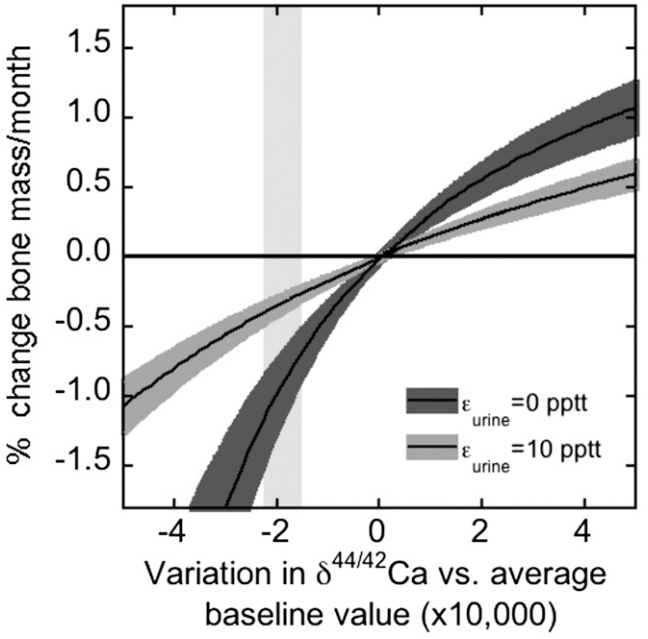

Fig. 3. Estimating changes in BMB from variations in $\delta^{44/42}$Ca using the Ca isotope model. The curved fields indicate predicted percentage bone loss per month for subjects as a function of the shift in $\delta^{44/42}$Ca of urine from the baseline value. Predictions are shown for two different values of renal fractionation factor ($\varepsilon^{44/42}Ca_{urine}$; abbreviated here as ε_{urine}): 0 and 10 pptt. The width of the shaded fields indicates the combined 1σ uncertainty of the Ca isotope model. The gray vertical box indicates the observed mean baseline-normalized difference in abundance of $\delta^{44/42}$Ca during bed rest for all subjects in this study. The width of this box indicates 2 SE widths about the mean. The conversion from milligrams Ca per day to percentage bone loss is (mg Ca/d) × (30 d/mo) × (1 g/1,000 mg)/(1,000 g Ca/person) × 100%.

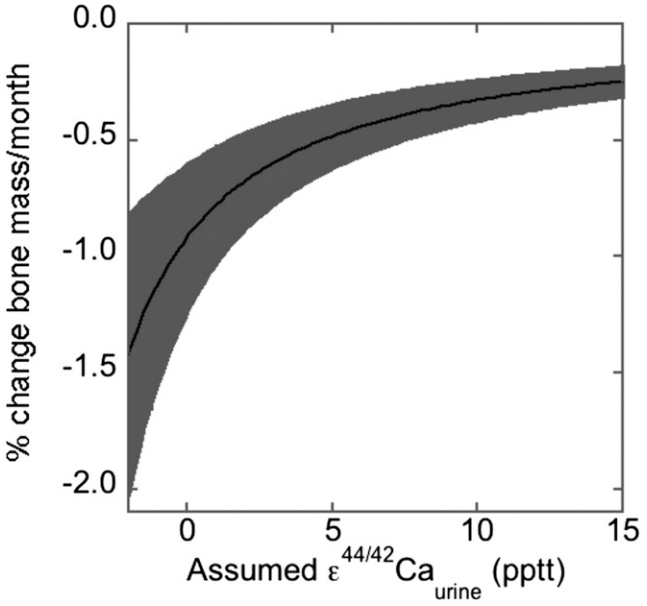

Fig. 4. Sensitivity of the Ca isotope model to the value assumed for the renal fractionation factor ($\varepsilon^{44/42}Ca_{urine}$) for the conditions of our study. The black line represents the model output assuming the following: a Ca isotope shift during bed rest of −2.0 ± 0.4 pptt, a value for the renal fractionation given by the *x* axis, and all other values and uncertainties as given in the text. The gray shaded area represents the model uncertainty determined by formal propagation of the uncertainty in the model parameters and Ca isotope measurements using the partial derivatives of all model equations with respect to the input variables. Estimates of the Ca isotopic offset between urine and blood in humans (6) and measurements of this offset in sows reported here (Table S2) indicate that the most likely value for human renal fractionation is about 10 pptt. As long as this value is >5 pptt, the exact value of this parameter does not significantly alter our estimates of bone loss during bed rest.

Using this model, we estimate that subjects lost on average 0.25 ± 0.07% (1 SD) of their bone mass from days 7 to 30 of bed rest (~109 mg Ca/d), assuming a renal isotopic fractionation of 10 pptt. The estimated error includes all measurement and model uncertainty, except for the uncertainty in renal isotopic fractionation. This rate of bone loss extrapolates to a loss of 1.0 ± 0.3% of skeletal mass over 90 d, which is equivalent, within error, to bone loss rates determined by X-ray densitometry scans in long-term bed rest studies (14, 22, 32–34). Thus, the Ca isotope model used here yields quantitative results that are consistent with the best existing measures of changes in BMD.

Conclusion. Measurement of naturally occurring Ca isotopes has the potential to reduce the duration of experimental studies of bone metabolism, accelerating the pace of discovery of new treatments for metabolic bone disease. The Ca isotope technique also promises to provide insights into the short-term dynamics of bone metabolism. Research into renal and hepatic Ca isotope fractionation will increase this use by refining our knowledge of the quantitative relationship between Ca isotopes and BMB. In the future, the Ca isotope technique may be useful in clinical settings to allow close monitoring of subjects at risk for bone loss and safe, rapid assessment of individual subjects' response to treatment.

Materials and Methods

Experimental Methods. The bed rest study was conducted at the University of Texas Medical Branch at Galveston's Institute for Translational Sciences—Clinical Research Center. Bed rest conditions were rigorously controlled, including room temperature and participant sleep and wake cycles. Energy intake was adjusted so that each participant maintained a constant weight (±3% of weight on bed rest day 3). Apart from this adjustment, participants were fed the same diet consisting of the same meals rotated on a 10-d cycle. Because participants entered the study on different calendar days, the diet cycle does not correspond to different study days. During bed rest, participants were confined to a strict −6° head-down tilt bed rest. They were monitored to ensure round-the-clock compliance. Details have been reported elsewhere (34–36). In this study, 12 subjects (8 male subjects and 4 female subjects) with an age range of 25–49 y and an average age of 32 ± 8 y were enrolled. All participants were of normal health with body mass indices between 20 and 30. All of the women were premenopausal. Demographic information for each subject is presented in Table S3. Details of participant recruitment and exclusion criteria are given elsewhere (35). Subjects began the ~54-d study at different times throughout a 6-mo period. The Johnson Space Center Committee for the Protection of Human Subjects and the University of Texas Medical Branch Institutional Review Board approved the study protocol. All subjects provided written, informed consent before they were enrolled.

Urine and blood samples were collected from all subjects, and food samples were collected from the 10-d meal rotation. The urine was acidified with trace metal-grade 20% HNO_3 and shipped to Arizona State University. Digestion, purification, and measurement of selected samples followed the method in the work by Morgan et al. (9). Details of chemical and mass spectrometric methods, and of the isotopic standard (ICP1) used in the present study, are provided in the *Supplemental Information*.

Mathematical Model. We derived a quasi-steady-state solution for the rate of bone loss during bed rest as a function of the shift in the Ca isotopic composition of urine (Fig. 2). In addition to Ca isotope fractionation during bone formation, the model allows for additional isotope fractionation during renal and hepatic (bile) excretion of Ca, which was proposed in the work by Heuser and Eisenhauer (6).

In this model, F_{diet} is the flux of calcium absorbed from diet, F_{bone} and F_{resorp} are the bone formation and resorption fluxes, and F_{urine} and F_{bile} are the net excretion fluxes of calcium through urine and bile, respectively. For simplicity, $\delta^{44/42}Ca$ is abbreviated to δ in equations. δ_{soft} and δ_{bone} are the Ca isotopic compositions of the soft tissue and bone pools, δ_{diet} and δ_{urine} are the isotopic compositions of the fluxes of Ca absorbed from diet and excreted in urine, and ε_{bone}, ε_{urine}, and ε_{bile} are the isotopic fractionations associated with forming bone, urine, and bile from the soft-tissue pool. For example, the isotopic composition of urine is given by Eq. **1**:

$$\delta_{urine} = \delta_{soft} + \varepsilon_{urine}. \quad [1]$$

We assume that subjects are in a state of long-term equilibrium before bed rest, such that there is no net change in bone mass and the isotopic composition of bone is in equilibrium with the soft tissue pool. The implication is that, before bed rest (as indicated by the superscript *i*), the fluxes and isotopic composition of absorbed and excreted calcium must be equal (Eq. **2**):

$$F_{diet} = F^{i}_{urine} + F_{bile} \quad [2]$$

and Eq. **3**:

$$\delta_{diet} = \frac{F^{i}_{urine}\delta^{i}_{urine} + F_{bile}(\delta^{i}_{urine} - \varepsilon_{urine} + \varepsilon_{bile})}{F^{i}_{urine} + F_{bile}} = \frac{F_{diet}\delta^{i}_{urine} + F_{bile}(\varepsilon_{bile} - \varepsilon_{urine})}{F_{diet}}. \quad [3]$$

Additionally, the isotopic composition of bone is given by Eq. **4**:

$$\delta_{bone} = \delta^{i}_{soft} + \varepsilon_{bone} = \delta^{i}_{urine} - \varepsilon_{urine} + \varepsilon_{bone} \quad [4]$$

When bed rest begins, we allow the rate of bone loss to increase, but we hold the dietary Ca flux and bone formation flux constant. Because the Ca content of soft tissue is tightly regulated, the Ca released from bone resorption during bed rest must be quickly excreted. This excretion happens primarily through the kidneys, where decreased Ca resorption during urine formation results in increased Ca excretion. We assume that the flux of hepatic Ca excretion flux via bile remains constant.

Because of the short residence time of Ca in soft tissue, the soft tissue Ca pool attains a state of quasiequilibrium after several days of bed rest. In this state, the mass and isotopic composition of soft tissue Ca is in equilibrium with the pool of bone Ca, which is slowly adjusting to bed rest on a timescale of years. During this period, we can write the following mass balance for soft tissue (Eq. **5**):

$$0 = F_{diet} - F_{bone} + F_{resorp} - F_{bile} - F_{urine} \quad [5]$$

and Eq. **6**:

$$0 = F_{diet}\delta_{diet} - F_{bone}(\delta_{soft} + \varepsilon_{bone}) + F_{resorp}\delta_{bone} - F_{bile}(\delta_{soft} + \varepsilon_{bile}) - F_{urine}(\delta_{soft} + \varepsilon_{urine}). \quad [6]$$

When we substitute Eqs. **1–5** into Eq. **6** and solve for F_{resorp}, all of the terms involving excretion of Ca in bile cancel, and therefore, we obtain Eq. **7**:

$$F_{resorp} = \frac{F_{bone}(\varepsilon_{bone} - \varepsilon_{urine}) + F_{diet}(\delta_{urine} - \delta^{i}_{urine})}{\varepsilon_{bone} - \varepsilon_{urine} - (\delta_{urine} - \delta^{i}_{urine})} = \frac{F_{bone}(\varepsilon_{bone} - \varepsilon_{urine}) + F_{diet}\Delta^{44/42}Ca_{baseline}}{\varepsilon_{bone} - \varepsilon_{urine} - \Delta^{44/42}Ca_{baseline}}, \quad [7]$$

where $\Delta^{44/42}Ca_{baseline} = \delta_{urine} - \delta^{i}_{urine}$. We report the relative rate of bone loss or gain relative to the mass of skeletal Ca, M_{bone}, as Eq. **8**:

$$BMB = \frac{F_{bone} - F_{resorp}}{M_{bone}}. \quad [8]$$

We use equation Eq. **7** to assess the sensitivity of our BMB estimate to uncertainties in the renal fractionation over a range of possible values from ε_{urine} = 0 to +15 pptt, with the most probable values near +10 pptt (Fig. 4). Errors in the estimate of the renal fractionation factor have minimal effect on estimates of BMB as long as this fractionation factor is greater than about 5 pptt.

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